CLAUDIA TIEMI MIYAMOTO ROSADA

Genotoxicidade dos antineoplásicos cisplatina (cis-diaminodicloroplatina, cis-DDP) e citosina-arabinosídeo (Ara-C), e do óleo essencial de *Eucalyptus globulus* através do ciclo parassexual em *Aspergillus nidulans*.

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas (área de concentração – Biologia Celular e Molecular) da Universidade Estadual de Maringá, para obtenção do grau de Doutor em Ciências Biológicas.

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Orientadora: ***Dra. Marialba A. A. de Castro-Prado***

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BIOGRAFIA

Claudia Tiemi Miyamoto Rosada, filha de Massayoshi Miyamoto e Eliza Ioshie Miyamoto, nasceu em Maringá-PR aos 12 de janeiro de 1978. Possui graduação em Farmácia-Bioquímica pela Universidade Estadual de Maringá-UEM (2001), especialização em Farmacologia pela UEM (2003) e Mestrado em Ciências da Saúde – Área de concentração em Doenças Infecciosas e Parasitárias, pela UEM (2005). Em abril de 2006, matriculou-se no curso de doutorado em Ciências Biológicas da Universidade Estadual de Maringá. Atualmente atua no grupo de pesquisa em Genética de Microorganismos da Universidade Estadual de Maringá.

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"Bom mesmo é ir à luta com determinação,
abraçar a vida com paixão, perder
com classe e vencer com ousadia,
pois o triunfo pertence a quem se atreve...
A vida é muito para ser insignificante."
Charles Chaplin.

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APRESENTAÇÃO

Esta tese, intitulada **Genotoxicidade dos antineoplásicos cisplatina (cis-diaminodicloroplatina, cis-DDP) e citosina-arabinosídeo (Ara-C), e do óleo essencial de *Eucalyptus globulus* através do ciclo parassexual em *Aspergillus nidulans***, é composta por dois artigos científicos:

1. **Genotoxicity (mitotic recombination) of the cancer chemotherapeutic agents cisplatin and cytosine arabinoside in *Aspergillus nidulans***. Publicado na Food and Chemical Toxicology, Estados Unidos, v. 45, p. 1091-1095, 2007. Fator de Impacto 2.186 (JCR 2007).

2. **Genotoxic activity of *Eucalyptus globulus* Essential Oil in *Aspergillus nidulans* Diploid Cells**, a ser enviado ao periódico Journal of Food Science, Estados Unidos, Fator de Impacto 1.255 (JCR 2007).

Genotoxicidade dos antineoplásicos cisplatina (cis-diaminodicloroplatina, cis-DDP) e citosina-arabinosídeo (Ara-C), e do óleo essencial de *Eucalyptus globulus* através do ciclo parassexual em *Aspergillus nidulans*.

Claudia Tiemi Miyamoto Rosada
Marialba Avezum Alves de Castro-Prado (Orientadora)

Resumo

Apenas uma pequena porcentagem de drogas sintéticas e espécies vegetais utilizadas na medicina tradicional e na medicina popular, respectivamente, foram adequadamente estudadas quanto a suas atividades farmacológicas e genotóxicas. Tais substâncias, através de seus prováveis efeitos mutagênicos, oncogênicos ou teratogênicos, podem produzir lesões ou rearranjos no DNA, possibilitando modificações no conteúdo informacional.

A carcinogênese é um processo extremamente complexo que resulta na transformação de uma célula normal em uma célula maligna, através de múltiplas alterações genéticas ou epigenéticas. Neste processo, genes que controlam a divisão e/ou a morte celular são alterados. Em células heterozigotas, alterações nos processos de recombinação homóloga e reparo do DNA podem induzir a carcinogênese, através da perda da condição de heterozigose cromossômica.

Os antineoplásicos citosina-arabinosídeo (Ara-C) e cis-diaminodicloroplatina (cis-DDP) são drogas utilizadas no tratamento de câncer. O mecanismo bioquímico responsável pela ação citotóxica de Ara-C é mediado pelo seu metabólito ativo Ara-CTP. Esse metabólito compete com a desoxicitidina trifosfato (dCTP) pela incorporação ao DNA, levando à pausa ou ao término da síntese de DNA, e gerando quebras na molécula e morte celular. A cis-DDP, por outro lado, reage com o DNA formando adutos bifuncionais, pois cada átomo de platina pode ligar-se a duas posições no DNA, gerando lesões intra- ou inter-cadeia, as quais após serem processadas, originam no DNA, quebras em ambas as cadeias. Alternativamente, o monoterpeno eucaliptol, caracterizado como principal constituinte do óleo essencial de *Eucalyptus globulus*, atua no DNA de células eucarióticas induzindo fragmentação na molécula e inibindo sua síntese. O óleo de *E. globulus*, com propriedades odorífera e antimicrobiana, é amplamente utilizado nas indústrias farmacêutica, de alimentos e de cosméticos. O fungo filamentoso *Aspergillus nidulans* é um excelente organismo para conduzir estudos de recombinagênese, pois passa a maior parte de seu ciclo celular em G2. Nesta fase, os cromossomos apresentam-se duplicados, o que favorece significativamente o evento de recombinação mitótica. O *crossing-over* mitótico, considerado um processo de ampla atuação em células diplóides eucariotas, ocorre durante a segregação das cromátides irmãs na mitose.

Em células heterozigotas, o *crossing-over* mitótico, através da segregação de uma cromátide paternal e uma recombinante para o mesmo pólo mitótico, conduz à homozigose todos os genes localizados em posição distal ao ponto de permuta. Tal homozigoze pode ter efeito carcinogênico pela redução da heterozigozidade constitucional de genes supressores de tumores. Desta forma, substâncias que induzem quebras no DNA, ou que inibem sua replicação, podem apresentar atividade carcinogênica por estimularem a ocorrência do *crossing-over* mitótico.

Considerando-se que os antineoplásicos Ara-C e cis-DDP e o óleo essencial de *E. globulus* promovem alterações no ciclo celular, o presente trabalho avaliou o efeito genotóxico destes compostos, em células diplóides heterozigotas de *A. nidulans*. Concentrações não-citotóxicas de Ara C (0,2 µM, 0,4 µM e 0,8 µM) e cis-DDP (1,5 µM, 3,0 µM e 6,0 µM) foram utilizadas para a avaliação do potencial recombinagênico destas drogas anticancerígenas, através da determinação dos Índices de Homozigotização (HI) para diferentes marcadores nutricionais. Este índice avalia a indução de homozigose em genes inicialmente presentes em heterozigose, através do *crossing-over* mitótico, sendo definido como a razão entre o número de segregantes prototróficos e o número de segregantes auxotróficos. O diplóide UT448//A757, heterozigoto para vários marcadores nutricionais, foi cultivado em Meio Mínimo + Ara C e Meio Mínimo + cis-DDP. O tratamento em Meio Mínimo permite apenas a seleção de diplóides prototróficos heterozigotos (+/- ou -/+) ou homozigotos (+/+), visto que diplóides auxotróficos (-/-) não crescem neste meio de cultura. Desta forma, se os compostos em análise induzirem o *crossing-over* mitótico, os valores de HI serão iguais ou superiores a 2,0 (4+: 2-). Por outro lado, na ausência de *crossing-over*, os valores do HI serão iguais ou inferiores a 1,0 (4+: 4-). Para o diplóide A757//UT448 foram determinados os valores de HI para os genes *paba, bi, met, piro e ribo* (ácido *p*-aminobenzóico, biotina, metionina, piridoxina e riboflavina respectivamente). Células diplóides de *A. nidulans* tratadas com 0,4 µM e 0,8 µM de Ara-C mostraram valores de HI significativamente superiores aos valores do controle, para todos os marcadores nutricionais. Para os diplóides tratados com cis-DDP, os valores de HI obtidos foram significativamente superiores aos do controle para as três concentrações testadas. Estes resultados demonstram o potencial recombinagênico de ambos agentes antineoplásicos. Considerando-se que células heterozigotas para genes supressores de tumor (*m*/+) podem originar clones de células homozigotas para o alelo não-funcional (*m*/*m*), através da recombinação mitótica ou somática, os antineoplásicos Ara C e cis-DDP podem ser

caracterizados como promotores no processo carcinogênico, sendo potencialmente capazes de induzir malignidades secundárias em pacientes com câncer, após o tratamento quimioterápico.

O potencial recombinagênico do óleo de *E. globulus* foi também avaliado no presente estudo, utilizando-se o ensaio de segregação somática e a linhagem diplóide A757//UT448 de *A. nidulans,* heterozigota para dois marcadores de coloração de conídios: *wA2* (conídios brancos) e *yA2* (conídios amarelos). Esta linhagem quando cultivada em MC origina segregantes mitóticos (ou setores) haplóides e diplóides, brancos e amarelos, os quais podem ser identificados por inspeção visual nas colônias do diplóide, cujos conidios são verdes. Os setores coloridos foram isolados, purificados e inoculados em MC suplementado com benomyl (0,5 µL/mL), para a avaliação da ploidia dos segregantes. Os segregantes que formaram colônias estáveis mitoticamente em presença de benomyl (agente haploidizante) foram classificados como haplóides. Por outro lado, os segregantes que originaram novos setores mitóticos em presença do agente haploidizante foram classificados como diplóides recombinantes. Segregantes aneuplóides foram também identificados nestas análises, através das seguintes características fenotípicas: produção de esporos e crescimento micelial reduzidos, e acentuada instabilidade mitótica. Os segregantes coloridos (amarelos e brancos) foram isolados dentre 100 colônias do diplóide A757//UT448, após tratamento com 0,0625 µL/mL, 0,125 µL/mL and 0,25 µL/mL do óleo de *E. globulus*. Os valores de MSI (razão entre o número de recombinantes em 100 colônias tratadas e o número de recombinantes em 100 colônias não-tratadas) foram posteriormente determinados e utilizados como critério para avaliar a genotoxicidade do óleo.

Entre os segregantes haplóides, os valores de MSI obtidos não foram estatisticamente significativos em relação ao controle, nas três concentrações analisadas. Por outro lado, o tratamento da linhagem A757//UT448 com duas das três concentrações de óleo selecionadas (0,125 µL/mL e 0,25 µL/mL) resultou em um aumento significativo no número de recombinantes diplóides, quando comparado com o controle. Esses dados demonstram que o óleo essencial de *E. globulus* pode atuar como um indutor de recombinação mitótica e originar clones de células homozigotas para genes deletérios, a partir de células diplóides heterozigotas. Os resultados também apontam para a necessidade de se avaliar a genotoxicidade deste composto em células de mamíferos, uma vez que o óleo em análise é amplamente utilizado nas indústrias de alimentos e farmacêutica.

Genotoxicity of anticancer cisplatin (cis- diamminedichloroplatinum, cis-DDP) and cytosine-arabinoside (Ara-C), and the essential oil of *Eucalyptus globulus* through parassexual cycle in *Aspergillus nidulans.*

Claudia Tiemi Miyamoto Rosada
Marialba Avezum Alves de Castro-Prado (Adviser)

Abstract

Only a small percentage of synthetic drugs and vegetal species used respectively in traditional medicine and in folk medicine has been sufficiently investigated for their pharmacological and genotoxic effects. These compounds may produce lesions or DNA rearrangements, including changes in information contents, through their probable mutagenic, oncogenic or teratogenic effects.

Carcinogenesis is a highly complex process and causes the transformation of a normal cell into a malignant one through multiple genetic or epigenetic changes. Genes that control division and/or the cell's death in such a process are changed. In heterozygous cells changes in the homologous recombination processes and DNA repairs may cause carcinogenesis by means of losses in the chromosome heterozygous condition.

Anticancer cytosine arabinoside (Ara-C) and cis-diamminedichloroplatinum (cis-DDP) are drugs used in cancer treatment. The biochemical mechanism for cytotoxic activities in Ara-C is mediated by its active metabolite Ara-CTP. The latter competes with deoxycytidine triphosphate (dCTP) for DNA incorporation, leads towards a pause or a stop in DNA synthesis, producing breaks in the molecule and causing cell death. On the other hand, since cis-DDP reacts to DNA and forms bi-functional adducts, each platinum atom may link itself to two positions in the DNA. Intra- and inter-chain lesions are produced which, after processing, cause breaks in both chains in the DNA. Alternatively, the monoterpene eucalyptol, the main compound of the *Eucalyptus globulus*'s essential oil acts on the eukaryotic cells' DNA and leads towards the fragmentation of the molecule and synthesis inhibition. *E. globulus* oil, with odiferous and antimicrobial features, is widely used in the pharmacy, food and cosmetics industries.

Due to the fact that the greatest part of the cell cycle of the filamentous fungus *Aspergillus nidulans* is passed in G2, it is a highly important organism for recombinogenesis studies. Chromosomes are duplicated at this phase and significantly provide mitotic recombination to the event. Since mitotic crossing-over may occur in eukaryotic diploid cells,

it occurs during the segregation of sister chromatids in mitosis. Mitotic crossing-over in heterozygous cells may conduce to the homozygous condition all the genes located at the distal position of the exchange site. This occurs through the segregation of a paternal chromatid and a recombinant for the same mitotic pole. Homozygosity may have a carcinogenic effect owing to a decrease in the constitutional heterozygosity of tumors suppressor genes. Compounds that cause breaks in DNA or which inhibit its replication may manifest carcinogenic activity due to the stimulation of mitotic crossing-over.

Since anticancer drugs Ara-C and cis-DDP, and the *E. globulus* essential oil cause changes in the cell cycle, current research evaluates the genotoxic effect of these compounds in heterozygous diploid cells of *A. nidulans*.

Non-cytotoxic concentrations of Ara-C (0.2 μM, 0.4 μM and 0.8 μM) and cis-DDP (1.5 μM, 3.0 μM and 6.0 μM) evaluated the recombinogenic potential of the above mentioned anticancerigenous drugs by determining the Homozygotization Index (HI) for different nutritional markers. HI evaluates homozygous induction in genes initially present in heterozygous condition through mitotic crossing-over. It may de defined as the ratio between the number of prototrophic segregants and the number of auxotrophic ones. Diploid UT448//A757, which is heterozygous for several nutritional markers, was cultivated in Minimum Medium + Ara-C and Minimum Medium + cis-DDP. Treatment in Minimum Medium merely selects heterozygous (+/- or -/+) or homozygous (+/+) prototrophic diploids since auxotrophic diploids (-/-) do not grow in the above culture medium. If the compounds under analysis induce mitotic crossing-over, HI rates will be either equal to or higher than 2.0 (4+:2-). On the other hand, HI rates in the absence of crossing-over will be equal to or lower than 1.0 (4+:4-). In the case of diploid A757//UT448, HI rates were determined for genes *paba*, *bi*, *meth*, *pyro* and *ribo* (respectively p-amino-benzoic acid; biotin; methionine; pyridoxine; and riboflavin).

Diploid cells of *A. nidulans* treated with 0.4 μM and 0.8 μM of Ara-C showed HI rates significantly higher than those of controls for all nutritional markers. HI rates for diploids treated with cis-DDP were significantly higher than those of control for the three concentrations under analysis. Results show the recombinogenic potential of both anti-neoplastic agents. Since heterozygous cells for tumor suppressor genes (*m*/+) may produce clones of homozygous cells for non-functional allele (*m/m*) through mitotic or somatic recombination, anti-cancer Ara-C and cis-DDP may be characterized as promoters of the

carcinogenic process and potentially able to induce secondary diseases in cancer patients after chemotherapy.

The recombinogenic potential of *E. globulus* oil was also evaluated by somatic segregation assay and diploid strain A757//UT448 of *A. nidulans*, which is heterozygous for two conidia color markers: *wA2* (white conidia) and *yA2* (yellow conidia). Haploid and diploid, white and yellow, mitotic segregants are produced when strain is cultivated in Complete Medium (CM). They may be visually identified in diploid colonies with green conidia. Color sectors were isolated, purified and inoculated in CM supplemented with benomyl (0.5 µL/mL) to evaluate segregants' ploidy. Segregants that produced mitotically stable colonies in benomyl (a haploidizing agent) were classified as haploids. On the other hand, segregants that produced new mitotic sectors in the presence of the haploidizing agent were classified as recombinant diploids. Aneuploid segregants were also identified in current analyses by means of the following phenotypes: reduced mycelial growth and high mitotic unstableness. Colored segregants (yellow and white) were isolated in 100 colonies of diploid A757//UT448 after treatment with 0.0625 µL/mL; 0.125 µL/mL and 0.25 µL/mL of *E. globulus* oil. MSI values (ratio between the number of recombinants in 100 treated colonies and the number of recombinants in 100 non-treated colonies) were later determined and used as an evaluation criterion of oil genotoxicity.

MSI values among haploid segregants were statistically non-significant in the three concentrations analyzed when compared to those of control. On the other hand, treatment of strain A757//UT448 with two out of three selected oil concentrations (0.125 µL/mL and 0.25 µL/mL) caused a significant increase in the number of recombinant diploids when compared to those of control. Data show that *E. globulus* essential oil may induce mitotic recombination and give rise to homozygous cell clones for deleterious genes from heterozygous diploid cells. Results also suggest that the genotoxicity of the compound in mammals' cells should be evaluated since oil is widely used in food and pharmaceutical industries.

Genotoxicity (mitotic recombination) of the cancer chemotherapeutic agents cisplatin and cytosine arabinoside in *Aspergillus nidulans*.

Genotoxicity (mitotic recombination) of the cancer chemotherapeutic agents cisplatin and cytosine arabinoside in *Aspergillus nidulans*.

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Key words: antineoplastic agents, tumor promoters, somatic recombination, second malignances.

Running title: Gene homozygosis induced by anticancer drugs

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ABSTRACT

Cisplatin (cis-diamminedichloroplatinum, cis-DDP) and cytosine arabinoside (Ara-C) are anticancer drugs used in the treatment of human cancer. The two chemotherapeutic drugs were tested in current research for their recombinogenic potential in diploid cells of *Aspergillus nidulans*. Non-cytotoxic concentrations of Ara-C (0.4 and 0.8 μM) and cis-DDP (1.5, 3.0 and 6.0 μM) were strong recombinagens in *A. nidulans* UT448//A757 diploid strain, which induced homozygosis of recessive genetic markers, previously present in heterozygous condition. Drugs significantly increased Homozygosity Index (HI) values for five nutritional genetic markers when compared with those determined in the absence of anticancer drugs. Since mitotic recombination is a mechanism leading to malignant growth through loss of heterozygosity at tumor-suppressor loci, Ara-C and cis-DDP may be characterized as secondary promoters of malignant neoplasia in diagnosed cancer patients, after chemotherapy treatment.

1. INTRODUCTION

The development of human neoplasia requires multiple genetic alterations followed by clonal expansion. Some alterations, such as the loss of tumor-suppressor gene function, are recessive and will not be expressed if present as single copies in heterozygous diploid cells. Further, somatic alterations that inactivate the remaining normal allele may occur with a subsequent loss of heterozygosity (LOH). LOH process leads to tumorigenesis due to the cellular absence of a functional tumor-suppressor gene (Imreh et al., 2003; Tischfield, 1997).

Mechanism leading to LOH may include chromosomal nondisjunction, mitotic recombination, gene conversion or even an epigenetic inactivation of the functional allele in a heterozygous cell (Young et al., 2006; Weinberg, 1991). The somatic recombination process in retinoblastoma (*Rb*) locus may contribute to as many as 75% of LOH events (Lasko et al., 1991).

Mitotic recombination in the G2 phase of the cell cycle consists of genetic exchanges between homologous DNA sequences. The process, posterior to chromosome segregation and cell

division, may originate homozygosis of distal genes to the point of exchange. Mitotic recombination is an essential mechanism for faithful DNA replication in vertebrate cells, besides being an important repair pathway of double-strand breaks (Helleday, 2003; Galli and Schiestl, 1998). Recombinational repair of chromosomal double-strand breaks may results in LOH (Moynahan and Jasin, 1997).

Mitotic recombination may be caused by DNA damaging agents and special *Aspergillus nidulans* diploid strains have been used to test the recombinogenic potential of chemical substances (Chiuchetta and Castro-Prado, 2005; Becker et al., 2004). *A. nidulans* is a filamentous fungus which has a well-characterized genetic system and whose cells pass the great part of their cell cycle in G2 phase (Bergen and Morris, 1983). This eukaryotic organism is, therefore, an excellent model system for investigating fundamental cell processes such as mitotic recombination.

Cisplatin (cis-diamminedichloroplatinum, cis-DDP) and cytosine arabinoside (Ara-C) are drugs with a therapeutic activity against human cancer and Ara-C is a nucleoside analogue used in the treatment of acute leukemia (Grant, 1998; Yarema et al., 1995). The biochemical mechanism responsible for the cytotoxic action of Ara-C is mediated by its active metabolite triphosphate Ara-CTP, generated by deoxycitidine kinase. Ara-CTP competes with deoxycytidine triphosphate (dCTP) for DNA incorporation and terminates or pauses the DNA synthesis at the site of incorporation, causing DNA strand breakage and cell death (Kufe et al., 1980). Ara-C neurotoxicity has been recently explained to be a result from reactive oxygen radicals generation that cause oxidative DNA strand breaks and initiates the p-53-dependent apoptotic process (Geller et al., 2001).

Cisplatin (cis-DDP), a broad activity antineoplastic agent, especially useful in testicular and ovarian cancer treatment, reacts with DNA, forming both intrastrand and interstrand cross-links. Processed DNA adducts formed by cis-DDP yield double strand breaks as an intermediate lesion,

while excision and recombinational repair pathways are probably required to remove these lesions (Jones et al., 2006; Frankenberg-Schwager et al., 2005; Lo et al., 2003; Larminat and Bohr, 1994).

Since DNA double-strand breaks initiate crossing-over in eukaryotic organisms (Galli and Schiestl, 1998), current *in vivo* study examines anticancer agents Ara-C and cis-DDP's potential in inducing somatic recombination in a diploid strain of *A. nidulans*. Loss of heterozygosity of nutritional markers is evaluated by Homozygosity Indexes which detect the homozygotization of recessive genes previously present in heterozygous conditions.

2. MATERIALS AND METHODS

2.1. *Fungal strains*

A. nidulans strains were derived from Utrecht stock (UT448) and from Fungal Genetic Stock Center (A757) (Table 1).

Table 1. Genotype and origin of *A. nidulans* strains

Strains	*Genotype*	*Origin*
A757	*yA2* (I), *methA17* (II), *pyroA4* (IV).	FGSC*
UT448	*riboA1* (I), *pabaA124*(I), *biA1*(I), *AcrA1* (II), *wA2*(II),	Utrecht, Holand

Requirements for: riboflavin = *ribo*A1, p-aminobenzoic acid = *paba*A124, biotin = *bio*A1, methionine = *meth*A17, pyridoxine = *pyro*A4. Conidia color: white = *wA2*; yellow =*yA2*. *AcrA1*, resistance to acriflavine.*FGSC = Fungal Genetic Stock Center, University of Kansas Medical Center, Kansas, USA.

2.2 *Culture media.*

2.2.1 Minimal Medium (MM): consisted of demineralized water (900.0 mL), concentrated MM solution (100.0 mL): $NaNO_3$ (60.0 g), KH_2PO (15.2 g), $MgSO_4 . 7 H_2O$ (5.2 g), KCl (5.2 g),

$FeSO_4$. 7 H_2O (0.02 g), $ZnSO_4$. 7 H_2O (0.01 g), $CuSO_4$. 5 H_2O (0.01 g), glucose (10.0 g), pH 6.0. To prepare solid MM, 15.0 g of Difco bacto Agar was added.

2.2.2 Complete Medium (CM): consisted of peptone (2.000 mg/L), yeast extract (2.000 mg/L), hydroliysed casein (1.000 mg/L), glucose (10.000 mg/L), biotin (2 mg/L), pyridoxine (50 mg/L), p-aminobenzoic acid (50 mg/L), folic acid (50 mg/L), nicotinic acid (100 mg/L), pantothenic acid (200 mg/L), choline chloride (200 mg/L), riboflavin (100 mg/L), inositol (400 mg/L) and thiamine (50 mg/L) added to MM.

2.2.3 Selection Medium (SM): comprised MM plus riboflavin (0,1 µg/mL), p-aminobenzoic acid(0,6857 µg/mL), biotin(0,02 µg/mL), methionine (149,21 µg/mL) and pyridoxine(0,05 µg/mL), while solid medium contained 1.5% agar. Incubation for strain growth occurred at 37ºC.

2.3 *Obtaining the heterokaryon and isolation of diploid strain.*

Heterokaryon-obtaining technique consists of placing two genetically complementar conidia strains (UT448 and A757) in 2.5 mL of MM supplemented with to 2% CM, incubated at 37ºC during four days. CM triggers a discrete development of the conidia and facilitates the hyphae's anastomosis. The formation of a compact mycelial mass, called vegetative heterokaryon, occurs after 72 h. It contains two different types of nuclei in a common cytoplasm. Mycelial mass was then transferred in petri plates with MM and incubated at 37ºC during five days. At the end of this period sectors, strongly growing and featuring green conidia, were reported in the heterokaryotic colony (Fig. 1a). Green conidia sectors were transferred to new plates with MM and incubated at 37ºC for five days. MM growth corroborates the diploid prototrophic characteristics of the green conidia sectors (Roper, 1952).

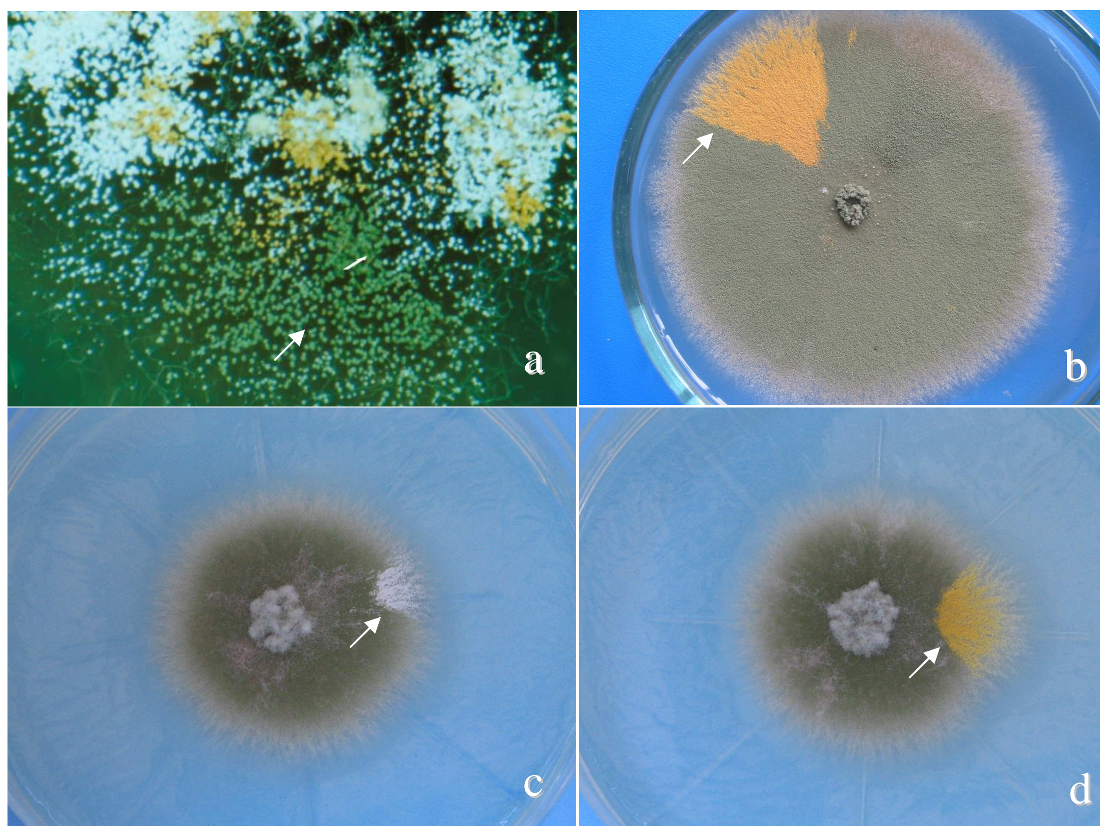

Fig. 1 (a) Origin of the UT448//A757 diploid strain (arrow) from the heterokaryon formed between the haploid UT448 (with white conidia) and A757 (with yellow conidia) strains. (b) Growth of the UT448//A757 diploid strain in the presence of 0.8 μM (D4) of cytosine arabinoside. (c) Diploids D8 and (d) D9 obtained after treatment of UT448//A757 diploid strain with 3.0 μM (D8) and 6.0 μM (D9) of cis-DDP. Arrows indicate the origin of diploid.

2.4. Ara-C *and* cis-DDP *treatment.*

Filter-sterilized aqueous solutions of Ara-C ($C_9H_{13}N_3O_5$, FW 243.3, Sigma-Aldrich Co., Catalog # C1768, 100% pure) and cis-DDP ($Pt(NH_3)_2Cl_2$, FW 300.1, Sigma-Aldrich Co., Catalog # P4394, 100% pure) were added to molten minimal medium. Non-cytotoxic concentrations of Ara C (0.2 μM, 0.4 μM and 0.8 μM) and cis-DDP (1.5 μM, 3.0 μM and 6.0 μM) were used in the present study for the recombinogenesis tests. In the case of toxicity measurements, colonies' diameters were determined during six days after incubation. Rates in the presence and in the absence (control) of drugs were compared by Student's t test ($p < 0.05$) (results not shown).

2.5 *Haploidization of the diploid strains obtained after treatment with* Ara-C *and* cis-DDP.

Colonies of diploid *A. nidulans* strain were grown onto petri plates containing MM (control), MM + Ara-C (0.2 to 0.8 μM) and MM+ cis-DDP (1.5 to 6.0 μM). Plates were incubated for six days at 37° C and then visually inspected for diploid color (white, yellow or green) sectors arising against a background of green conidia of the original diploid strain, which would indicate the occurrence of mitotic recombination. Diploid UT448//A757, grown in MM, spontaneously gives rise to a mitotic diploid sector with green conidia (D1, control). First treatment (MM + Ara-C) produced three morphologically identifiable diploid sectors, named D2 to D4. Second treatment (MM + cis-DDP) originated five diploid sectors which were sequentially numbered D5 to D9. Sectors were homozygous (+/+) or heterozygous (+/- or -/+) diploids but never recessive homozygotes (-/-) because the latter cannot grow on MM. The new diploid sectors (D1 to D9) were purified on MM, individually transferred to CM plates and processed by spontaneous haploidization. Haploidization, the loss of one member of each chromosome pair through successive mitotic divisions, results in the haploid condition of the nuclei. Each diploid sector (D1 to D9), after haploidization, originated haploid mitotic segregants, which were purified in CM and their mitotic stability evaluated in CM + benomyl (0.2 μg/mL). Benomyl is a fungicide used as a haploidizing agent of diploid strains of *A. nidulans*. The haploidization induced by benomyl is the result of chromosomal loss and mitotic non-disjunction (Hastie, 1970). Aneuploid cells (2n – 1; 2n -2, etc), produced at this stage, are highly unstable and randomly lose chromosomes till the haploid stage is finally reached.

Only mitotically stable segregants defined at the final stage were selected for the recombinogenesis test (Chiuchetta and Castro-Prado, 2005). Conidia from each haploid sector were individually transferred to 25 defined positions (5X5 pattern) on CM plates and their nutritional requirements were determined: MM supplemented with all nutritional requirements of the UT448 and UT196 strains, excepting one, in each medium type.

2.6 *Analysis of mitotic crossing-over by determination of the homozygosity index*

Mitotic crossing-over causes homozygotization of heterozygous-conditioned genes. If the antineoplastic agents induce mitotic crossing-over in diploid strain UT448//A757, only prototrophic heterozygotes (+/- or -/+) or homozygotes (+/+) diploids will develop in MM and the nutritional markers will segregate among the haploids in the proportion of 4+ to 2-. However, if the drugs fail to induce crossing-over, the proportion will be 4+ to 4- because the initial selection process limits the growth of auxotrophic diploids (-/-). The ratio of prototrophic to auxotrophic segregants is described by the Homozygosity Index (HI) or rather, an HI equal to or higher than two indicates recombinogenic effects of anticancer drugs. The recombinogenic potential of the drugs was assessed by comparing the homozygotization indices of the nutritional markers with Yates Corrected Chi-square test, $p < 0.05$.

3. RESULTS

The treatment of *A. nidulans* diploid cells with Ara-C middle (0.4 μM) and high (0.8 μM) concentrations increased HI values for *ribo*, *paba*, *bi*, *meth* and *pyro* markers when compared to control HI values (Table 2). On the other hand, analyses of HI values, obtained by low dose Ara-C (0.2 μM), were not significantly different from HI controls (results not shown).

Since Ara-C and cis-DDP treatments of UT448//A757 strain in MM impaired the growth of auxotrophic diploids, most Ara-C- and cis-DDP-induced diploids were heterozygous for the nutritional genetic markers. Treatments, however, did not limit the growth of homozygous diploids for recessive conidia color genes. Yellow (*y*//*y*) and white (*w*//*w*) diploid sectors were in fact distinguishable from the parental heterozygous strain (UT448//A757) growing in the presence of the chemotherapeutic drugs. Diploids D4 (yellow), D8 (white) and D9 (yellow)(Fig.1b-d) diploid strains were obtained with 0.8 μM Ara-C, 3.0 μM cis-DDP and 6.0 μM cis-DDP respectively.

Table 2. Homozygotization Index (HI) values for markers from UT448 // A757 diploid strain after treatment with 0.4 μM (D2 and D3) and 0.8 μM (D4) of cytosine arabinoside.

Markers[a]	**D1** (Control)[b]		**D2**		**D3**		**D4**	
	NS[c]	HI	NS[c]	HI	NS[c]	HI	NS[c]	HI
ribo+	156	1.3	118	**2.2***	60	1.8	37	0.8
Ribo	123		54		34		44	
paba+	151	1.2	114	**2.0***	62	1.9	37	0.8
Paba	128		58		32		44	
bi+	163	1.4	119	2.2	65	**2.2***	77	**19.3***
Bi	116		53		29		04	
meth+	154	1.1	157	**10.5***	87	**12.4***	80	**80.0***
Meth	135		15		07		01	
pyro+	138	1.0	75	**2.0***	54	1.8	41	1.0
Pyro	141		37		30		40	

[a] *ribo* = riboflavin; *paba*= *p*-aminobenzoic acid; *bi* = biotin; *meth* = methionine and *pyro* = pyridoxine. [b]Diploid D1, not treated with the antineoplastic drugs. [c]Number of mitotic segregants.*Significantly different from control ($p < 0.05$). nd= not determined (see text).

Phenotypic analyses of D4 and D8 diploids showed they were recombinant for *paba-y* and centromere-*meth* intervals, respectively (Tables 2, 3).

Contrastingly, the D9 diploid strain was incapable of growing on MM and showed to be phenotypically homozygous for *y* and *paba* genes. HI value for *paba* gene could not be determined due to the homozygous condition for this marker (*paba+//paba+*) in the D9 genome. Even HI value for the *meth* gene could not be determined in D9 strain due to the absence of segregants with recombinant phenotype (Tables 1 and 3).

In paternal UT448 haploid strain, the *w* gene is closely linked to the *meth*+ gene and, consequently, most of *w* segregants have the *w meth*+ phenotype. Eighty-one mitotic segregants with *w* phenotype were isolated from D4 diploid after its haploidization in CM. Only one was phenotypically characterized as *w meth* and classified as haploid stable recombinant (Tables 1, 2). On the other hand, HI value for *meth* gene could not be determined for D8 (white) diploid because *meth* segregation was not expected from homozygous *w meth*+//*w meth*+ diploid strain (Table 3).

4. DISCUSSION

Current study demonstrates the genotoxicity of two chemotherapeutic agents in *A. nidulans* diploid cells. Both Ara-C and cis-DDP anticancer drugs, in non-toxic concentrations, may be recognized as efficient agents in inducing somatic recombination and homozygosis of genetic markers in heterozygous diploid cells. There is growing evidence indicating that homologous recombination plays a role in different stages of carcinogenesis (Bishop and Schiestl, 2002; Lasko et al 1991).

The therapeutic efficacy of cis-DDP in human malignances is believed to result from the inhibition of DNA replication and transcription. Besides being genotoxic, DNA lesions formed by cis-DDP have been reported to induce homologous recombination in human colon carcinoma cells, proficient and deficient to DNA mismatch repair.

The cis-DDP human carcinogen also induces base transitions (GC→AT) and transversion (GC→TA and AT→TA) in human B-lymphoblasts, and chromosome aberrations in V79 Chinese hamster cells (Lin and Howell, 2006; Yarema et al., 1995; Cariello et al., 1992; Turnbull et al., 1979). The nucleoside analogue Ara-C has also been reported to induce recombination in *Drosophila melanogaster* as well as in mammalian cells. The Ara-C cytotoxicity is a direct result of interferences with semiconservative DNA synthesis leading to DNA strand breaks and chromosome fragmentation (Cunha et al., 2002; Arnaudeau et al., 2000).

Table 3. Homozygosity Index (HI) values for markers from UT448 // A757 diploid strain after treatment with 1.5 µM (D5 and D6), 3.0 µM (D7 and D8) and 6.0 µM (D9) of cisplatin.

Markers[a]	**D1** (Control)[b]		**D5**		**D6**		**D7**		**D8**		**D9**	
	NS[c]	HI	NS[c]	HI	NS[c]	HI	NS[c]	HI	NS[c]	HI	NS[c]	HI
ribo+	156	1.3	43	0.9	38	0.7	123	**2.0***	61	**2.4***	71	1.0
ribo	123		50		52		61		25		69	
paba+	151	1.2	39	0.7	31	0.5	124	**2.1***	60	**2.3***	140	nd
paba	128		54		59		60		26		0	
bi+	163	1.4	33	0.6	31	0.5	123	2.1	56	1.9	131	**14.6***
bi	116		60		59		61		30		09	
meth+	154	1.1	83	**8.3***	84	**14.0***	179	**17.9***	86	nd	139	nd
meth	135		10		06		10		0		01	
pyro+	138	1.0	40	0.8	42	0.8	117	1.8	61	**2.4***	65	0.9
pyro	141		53		48		67		25		75	

[a] *ribo* = riboflavin; *paba*= *p*-aminobenzoic acid; *bi* = biotin; *meth* = methionine and *pyro* = pyridoxine.[b]Diploid D1, not treated with the antineoplastic drugs. [c]Number of mitotic segregants. *Significantly different from control ($p < 0.05$). nd= not determined (see text).

It has been shown that inhibitors of DNA-synthesis, such as doxorubicin and 5-azacytidine, are potent inducers of homologous recombination, probably due to the generation of double-strand breaks (Becker and Castro-Prado, 2004; Cunha et al. 2002, Arnaudeau et al., 2000). Thus, the recombinogenic potentials of cisplatin and cytosine arabinoside may be associated with the recombinational repair of Ara-C and cis-DDP-induced DNA strand breaks (Frankenberg-Schwager et al., 2005; Nakayama et al., 2005).

Genetic alterations are necessary to change a normal cell into a malignant one. Chemical and physical agents may act as initiators of genetic irreversible alterations leading to the origin of heterozygous cells for oncogenic mutations. While homologous recombination renders the cell homozygous for a pre-existing mutation, heterozygous cells for a mutant tumor-suppressor gene may give rise to cell clones with altered phenotype, via mitotic crossing-over (Young et al., 2006). Since tumor-suppressor genes which confer negative regulatory controls on neoplasm cell transformation may be completely suppressed in recombinant cells, studies have been carried out to identify the recombinogenic potential of chemical substances (Chiuchetta and Castro-Prado, 2005, Kaneshima and Castro-Prado, 2005). Chemotherapeutic agents such as Ara-C and cis-DDP may promote carcinogenesis in pre-malignant cells and lead to the development of second malignances through the induction of mitotic recombination. Actually, numerous reports have been associated the effects of chemotherapeutic agents and the pathogenesis of second malignant neoplasms (Vancura et al., 2006; Aung et al., 2002; Yamada et al., 1999; Smith et al., 1994).

Second leukemia after multiagent chemotherapy, including cisplatin, has been reported in a 62-year-old male patient with colon adenocarcinoma (Gokel and Paydas 2002), in long-term survivors of osteosarcoma (Aung et al., 2002) and in bladder cancer patients (Theodore et al., 2002). The development of secondary lung adenocarcinoma after treatment with varying combination chemotherapies, including the anticancer drug cytosine arabinoside, was reported in two patients with primary non-Hodgkin's malignant lymphoma (Yamada et al., 1999).

The increasing number of second malignancies diagnosed in cancer patients after chemotherapeutic regimens indicates the need to submit these patients to ongoing physical investigation for the early detection and treatment of second malignancies. Moreover, efforts should be addressed to a) the obtaining more detailed knowledge of the genotoxic profile of the anticancer drugs, especially on their effects on the chromosomes' structural and numerical alterations, and b) the new antineoplastic agents' development without the long-term carcinogenic effects.

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5. REFERENCES

Arnaudeau, C., Tenorio Miranda, E., Jenssen, D. and Helleday, T., 2000. Inhibition of DNA synthesis is a potent mechanism by which cytostatic drugs induce homologous recombination in mammalian cells. Mut. Res. 461, 221-228.

Aung, L., Gorlick, R.G., Shi, W., Thaler, H., Shorter, N.A., Healey, J.H., Huvos, A.G., Meyers, P.A., 2002. Second malignant neoplasms in long-term survivors of osteosarcoma. Cancer. 95, 1728-1734.

Becker, T.C.A. and Castro-Prado, M.A.A., 2004. Parameiosis in *Aspergillus nidulans* in response to doxorubicin. Folia Microbiol. 49(6), 699-704.

Bergen, G.G. and Morris, N.R., 1983. Kinetics of the nuclear division cycle of *Aspergillus nidulans*. J. Bacteriol. 156, 155-160.

Bishop, A.J.R. and Schiestl, R.H., 2002. Homologous Recombination and its role in carcinogenesis. J. Biomed. Biotechnol. 2, 75-85.

Cariello, N.F., Swenberg, J.A. and Skopek, T.R., 1992. In vitro mutational specificity of cisplatin in the human hypoxanthine guanine phosphoribosyltransferase gene. Cancer Res. 52, 2866-2873.

Chiuchetta, S.J.R. and Castro-Prado, M.A.A., 2005. Genotoxic evaluation of sodium nitroprusside in *Aspergillus nidulans*. Genet. Mol. Biol. 28, 798-803.

Cunha, K.S., Reguly, M.L., Graf, U. and Andrade, H.H.R., 2002. Somatic recombination: a major genotoxic effect of two pyrimidine antimetabolotic chemotherapeutic drugs in Drosophila melanogaster. Mut. Res. 514, 95-103.

Frankenberg-Schwager, M., Kirchermeier, D., Greif, G., Baer, K., Becker, M., Frankenberg, D., 2005. Cisplatin-mediated DNA double-strand breaks in replicating but not in quiescent cell on yeast *Saccharomyces cerevisiae*. Toxicology 212, 175-184.

Galli, A. and Schiestl, R.H., 1998. Effects of DNA double-strand and single–strand breaks on intrachromosomal recombination events in cell-cycle-arrested yeast cells. Genetics. 149, 1235-1250.

Geller, H.M., Cheng, K.Y., Goldsmith, N.K., Romero, A.A., Zhang, A.L., Morris, E.J., Grandison, L., 2001. Oxidative stress mediates neuronal DNA damage and apoptosis in response to cytosine arabinoside. J Neurochem. 78, 265-275.

Gokel, Y. and Paydas, S., 2002. Chronic myeloid leukemia in a patient with colon adenocarcinoma. Haematologia 32, 501-503.

Grant, S., 1998. Ara-c: cellular and molecular pharmacology. Adv. Cancer Res. 72, 197-233.

Hastie, A.C., 1970. Benlate-induced instability of *Aspergillus* diploids**.** Nature 226, 771.

Helleday, T., 2003. Pathways for mitotic homologous recombination in mammalian cells. Mutat. Res. 532, 103-115.

Imreh, S., Klein, G., Zabarovskv, E.R., 2003. Search for unknown tumor-antagonizing genes. Genes Chromosomes Cancer. 38, 307-321.

Jones, E., Secord, A., Prosnitz, L.R., Samulski, T.V., Oleson, J.R., Berchuck, A., Clarke-Pearson, D., Soper, J., Dewhirst, M.W., Vujaskovic, Z., 2006. Intra-peritoneal cisplatin and whole abdomen hyperthermia for relapsed ovarian carcinoma. Int. J. Hyperthermia. 22, 161-172.

Kaneshima, E.N. and Castro-Prado, M.A.A., 2005. Benznidazole-induced genotoxicity in diploid cells of Aspergillus nidulans. Mem. Inst. Oswaldo Cruz 1000: 325-329.

Kufe, D.W., Major, P.P., Egan, E.M., Beardsley, G.P., 1980. Correlation of cytotoxicity with incorporation of ara-C into DNA. J. Biol. Chem. 255, 8997-9000.

Larminat, F. and Bohr, V., 1994. Role of the human ERCC-1 gene in gene-especific repair of cisplatin-induced DNA damage. Nucleic Acids Res. 22, 3005-3010.

Lasko, D., Cavenee, W., Nordenskjöld, M., 1991. Loss of constitutional heterozygosity in human cancer. Annu. Rev. Genet. 25, 281-314.

Lin, X. and Howell, S.B., 2006. DNA mismatch repair and p53 function are major determinants of the rate of development of cisplatin resistance. Mol. Cancer Ther. 5, 1239-1247.

Lo, T., Pellegrini, L., Venkitaraman, A.R., Blundell, T.L., 2003. Sequence fingerprints in BRCA2 and RAD51: implications for DNA repair and cancer. DNA Repair. 2, 1015-1028.

Moynahan, M.E. and Jasin, M., 1997. Loss of heterozygosity induced by a chromosomal double-strand break. Proc. Natl. Acad. Sci. U.S.A. 94, 8988-8993.

Nakayama, T., Sakamoto, S., Sassa, S., Suzuki, S., Kudo, H., Nagasawa, H., 2005. Paradoxical effect of cytosine arabinoside on mouse leukemia cell line L1210 cells. Anticancer Res. 25, 157-160.

Roper, J.A., 1952. Production of heterozygous diploids in filamentous fungi. Experientia 8, 14-15.

Smith, M.A., Rubinstein, L. and Ungerleider, R.S., 1994. Therapy-related acute myeloid leukemia following treatment with epipodophyllotoxins: estimating the risks. Med. Pediatr. Oncol. 23, 86-98.

Theodore, C., Bayle, C., Bernheim, A., Wibauld, P., 2002. Secondary leukaemia after treating advanced bladder cancer with methotrexate, vinblastine, doxirubicin and cisplatin chemotherapy. BJU Int. 90, 470-471.

Tischfield, J.A., 1997. Loss of heterozygosity or: How I learned to stop worrying and love mitotic recombination. Am. J. Hum. Genet. 61, 995-999.

Turnbull, D., Popescu, N.C., DiPaolo, J.A. and Myhr, B.C., 1979. Cis-platinum (II) diamine dichloride causes mutation, transformation, and sister-chromatid exchange in cultured mammalian cells. Mutat. Res. 66, 267-275.

Vancura, R.W., Kepes, J.J., Newell, K.L., Ha, T.M. and Arnold, P.M., 2006. Secondary intracranial neoplasms exhibiting features of astrocytoma and neuroblastoma in 2 children treated for acute lymphoblastic leukemia: report of 2 cases. Surg. Neurol. 65, 490-494.

Weinberg, R., 1991. Tumor suppressor genes. Science 254, 1138-1146.

Yamada, T., Shinohara, K., Takeda, K., Kamed, N., Katsuki, K., Ariyoshi, K. and Kamei, T., 1999. Second lung adenocarcinoma after combination chemotherapy in two patients with primary non-Hodgkin's lymphoma. Jpn. J. Clin. Oncol. 29: 226-228.

Yarema, K.J., Lippard, S.J. and Essigmann, J.M., 1995. Mutagenic and genotoxic effects of DNA adducts formed by the anticancer drug cis-diamminedichloroplatinum (II). Nucleic Acids Res. 23, 4067-4072.

Young, B.D., Debernardi, S., Lillington. D.M., Skoulakis, S., Chaplin, T., Foot, N.J., Raghavan, M., 2006. A role for mitotic recomnination in leukemogenesis. Advances in Enzyme Regulation 46, 90-97.

Genotoxic activity of *Eucalyptus globulus* Essential Oil in *Aspergillus nidulans* Diploid Cells.

Genotoxic activity of *Eucalyptus globulus* Essential Oil in *Aspergillus nidulans* Diploid Cells.

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Short version of title: Genotoxicity of *Eucalyptus* essential oil

Journal section: Toxicology and Chemical Food Safety

ABSTRACT

Essential oils from many *Eucalyptus* species are widely used in food, cosmetics, and pharmaceutical industries. In current research *Eucalyptus globulus* essential oil was evaluated for its genotoxic potential using a somatic segregation assay and a diploid strain of the fungus *Aspergillus nidulans*, heterozygous for nutritional and conidia color markers. The main compounds of current essential oil sample were eucalyptol (49.05%), α-pinene (8.89%), β-pinene (1.5%), globulol (6.95%), α-eudesmol (1.12%), spathulenol (1.42%), γ-cadinene (1.45%), trans-β-elemenone (1.23%) and aromandendrene (2.33%), totaling 73.9% of oil. Oil at 0.125 μL/mL and 0.25 μL/mL concentrations was found to increase the mitotic instability of the original A757//UT448 diploid strain, and the number of diploid mitotic recombinants. The oil's genotoxicity was associated with the induction of mitotic crossing-over or with oil-broken chromosomes.

KEY WORDS: Essential oil, Food Additives, Intergenic Recombination, Mitotic Segregation Index, Toxicity.

INTRODUCTION

Essential oils, also known as volatile or ethereal oils, are aromatic oily liquids obtained from flowers, bark, seeds, leaves, roots, or other plant elements. Essential oils, typically obtained by the method of steam distillation, have a wide application in folk medicine, food flavouring and preservation as well as in fragrance industries. Additionally, a large number of essential oils and their constituents have been investigated for their antibacterial and antifungal activities against human pathogen and food-related microorganisms. Biological activity of essential oils depends on their chemical compositions, which are determined by the plant genotypes and depend on various environmental factors (Silva and others 2003; Juergens and others 2003; Burt 2004; Sacchetti and others 2005; Ali and others 2008; Fisher and Phillips 2008; Viuda-Martos and others 2008).

The genus *Eucalyptus* comprises numerous species whose essential oils have been shown to exhibit analgesic, anti-inflammatory, and antipyretic properties for the treatment of respiratory disorders such as cold, bronchitis, and sinus congestion. The *E. globulus* essential oil, in particular, is extensively used as preservative and flavor additive in foods and beverages and as fragrance in cosmetics. The use of *E. globulus* oil in the food industry is based on its odorous and antimicrobial properties (Uemura and others 1997; Gomes-Carneiro and others 1998; Juergens and others 2003; Silva and others 2003). Actually, this essential oil, with its antimicrobial activity against human pathogens, such as *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Escherichia coli*, may be added as a flavor in a number of processed foodstuffs, such as baked goods, frozen dairy, soft candy, meat products and non-alcoholic beverages (De Vicenzi and others 2002; Cimanga and others 2002; Cermelli and others 2008).

The major constituent of *E. globulus* is eucalyptol (1,8-cineole), a monoterpene that induces DNA fragmentation and DNA synthesis inhibition in eukaryotic cells (Koitabashi and

others 1997; Moteki and others 2002). Chemical compounds that produce DNA-breaks or cause inhibition of the DNA synthesis may show carcinogenic activity due to the induction of mitotic recombination (Kaneshima and Castro-Prado 2005; Miyamoto and others 2007).

Mitotic recombination, occurring during the G2 phase of a heterozygous cell, consists of exchange events between homologous chromosomes that, following chromosome segregation and cell division, may result in the homozygosis of distal genes to the point of exchange. The process is called loss of heterozygosity (LOH). In heterozygous cells for a tumor suppressor gene, mitotic recombination may induce the loss of the functional tumor suppressor allele, which constitutes an important step for neoplastic transformation (Moynahan and Jasin 1997; Bishop and Schiestl 2002).

The p53 tumor-suppressor gene, previously characterized as a suppressor of the spontaneous homologous recombination, is frequently inactivated in human cancers by LOH (Stürzbecher and others 1996; Honma 2005). Furthermore, predisposed people to spontaneously increased levels of mitotic recombination, such as Bloom’s syndrome and Fanconi anemia patients, are actually prone to cancer (Festa and others 1979; Thyagarajan and Campbell 1997).

Since the carcinogenic potential of a chemical compound may be detected through its effect on mitotic recombination and since information available of the toxicity profile of the *Eucalyptus* essential oils is limited (Nielsen 2006), current research evaluated the recombinogenic potential of the oil of *E. globulus* using a somatic segregation assay and the filamentous fungus *Aspergillus nidulans*.

The life cycle of *A. nidulans* makes it a particularly useful organism for the monitoring of mitotic recombination. This is due to the fact that a substantial part of its cell-cycle is spent in G2, a period in which chromosomes are in duplicate and significantly favor mitotic recombination (Bergen and Morris 1983). Diploid strains of *A. nidulans* have in fact been used to detect the recombinogenic potential of several chemical compounds, such as the antiparasitic

drug benznidazole and the anticancer agent cisplatin, (Kaneshima and Castro-Prado 2005; Miyamoto and others 2007), which were also genotoxic in mammalian cells (Santos and others 1994; Jirkova and others 2006).

2. MATERIALS AND METHODS

2.1 *Strains and Media*:

Diploid strain A757//UT448 of *A. nidulans* (Miyamoto and others 2007) was used to estimate the *E. globulus* oil's recombinogenic potential. Diploid strain is heterozygous for two conidia color markers, *yA2* (yellow) and *wA2* (white), which allow direct visual detection of mitotic segregants as normally growing yellow or white sectors on the diploid green colonies (Figure 1). Standard Complete Medium (CM) employed was previously described by Miyamoto and others (2007).

2.2 *Plant material and extraction of essential oil*:

E. globulus leaves were collected in April 2000, in Curitiba PR Brazil (Unidade de Plantas Medicinais, Aromáticas e Condimentares, Parque Newton F. Maia), and identified by Dr. Cirino Correa Júnior (Emater Curitiba PR Brazil). After harvesting, 100 g of plant material, dried at temperatures ranging between 30 and 40 °C, were submitted to 6 h water-distillation, using a Clevenger-type apparatus to produce oil at 0.6% (v/w) yield. Oil samples were stored in amber flasks at 4 °C until tested and analyzed.

2.3 *GC-MS analysis:*

Mass spectra were determined by Varian 3800 Series GC-EIMS (SATURN 2000), 70 eV, capillary column CP-SIL 8 CB (30 m x 0.25 mm I.D. x 0.25 μm film thickness) with helium as carrier gas, at a flow rate of 1.0 mL/min; temperature schedule was 60 °C, increasing at the rate of 3 °C/min, to 90 °C; after 5 min, temperature was increased at the rate of 3 °C/min, to 140 °C; and, at the rate of 30 °C/min , to 240 °C, for 5 min at the latter temperature;

injection in the split mode (1:100) at an injector, at 250 °C; detector temperature at 200 °C; injection volume, 0.5 μL.

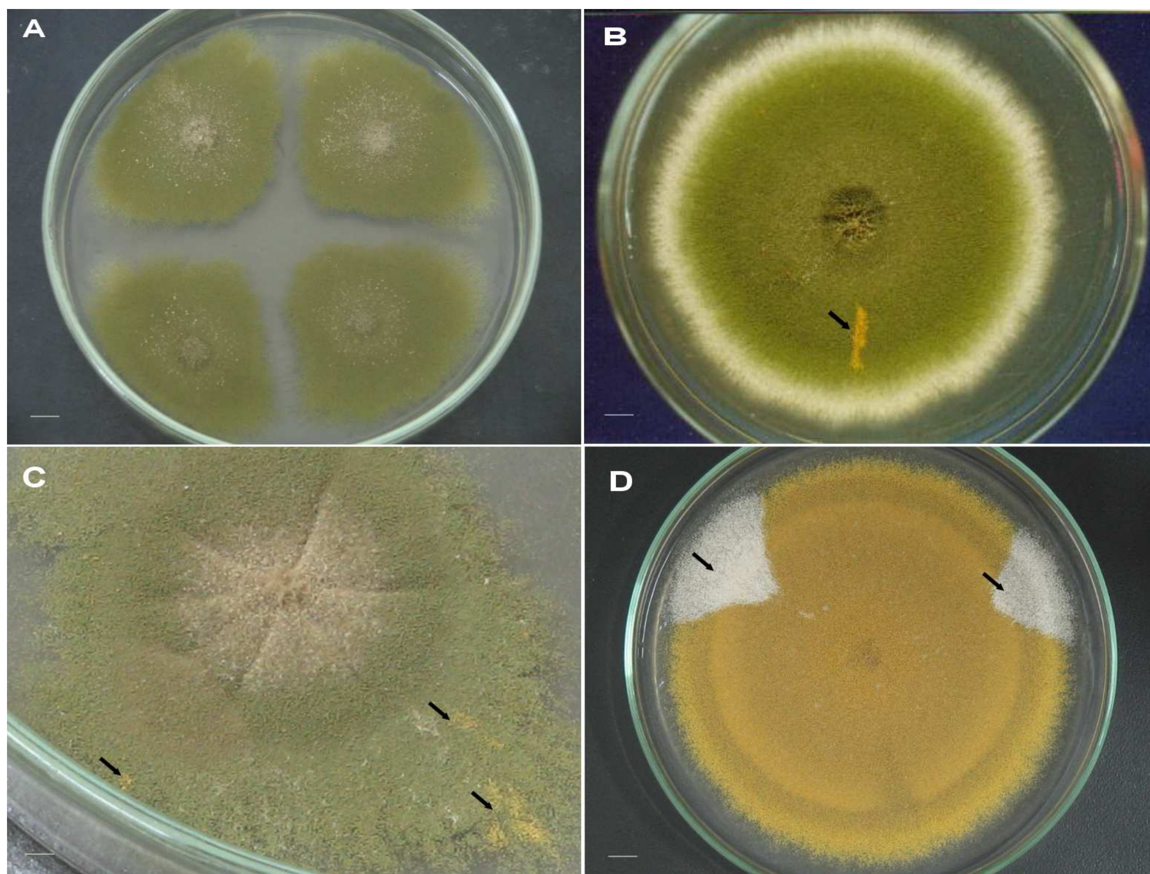

Figure 1. Growth of A757//UT448 diploid strain in the absence (A) and in the presence of 0.125 µL/mL (B) and 0.25 µL/mL (C) of the *E. globulus* essential oil. Diploid segregant derived from the A757//UT448 strain after treatment with 0.25 µL/mL *E. globulus* oil (D). Arrows indicate the origin of mitotic white and yellow segregants (sectors). Bars: 2 mm (A, D), 5mm (B) and 6mm (C).

Individual components were identified by comparing mass spectrum and their GC retention times (RT) and retention indices (RI) with those of authentic compounds previously analyzed and stored in the data system or found in the literature (Adams 1995). The NIST (National Institute for Standard Technology – 62,235 compounds) was used for the comparison of mass spectra. Retention indices were calculated for all volatile constituents using a homologous series (C_9 to C_{20}) recorded under similar operating conditions. Retention indices

(RI) have been obtained according to van den Dool and Kratz (1963) method and quantitative data were obtained by electronic integration of GC-FID (VARIAN CP 3800) peak areas.

2.4 *Evaluation of oil cytotoxicity*:

Conidia of diploid strain (A757//UT448) were inoculated in the center of CM (control) and CM + *E. globulus* oil (0.25 μL/mL and 0.5 μL/mL) plates. A total of five plates were inoculated and incubated at 37 °C. Colony diameters were measured with a ruler, once a day, during seven days. Results were analyzed with One-way Analysis of Variance and by Bonferroni post test, for $P< 0.05$.

2.5 *Calculating Mitotic Segregation Index*

Sterile aliquots of *E. globulus* oil were added to molten CM to obtain the desired concentrations for each test. Maximum concentration of *E. globulus* oil, which did not alter the colonies' morphology (0.25 μL/mL), and two lowest concentrations (0.0625 μL/mL, 0.125 μL/mL) were used to evaluate genotoxicity. Conidia of diploid strain A757//UT448 were inoculated at four equidistant positions of plates containing CM (control) and CM + *E. globulus* oil and (test). Plates were incubated at 37 ^{o}C, for six days. Afterward, colonies were inspected for the presence of white and yellow sectors growing on the green diploid colonies, which are indicators of the occurrence of mitotic segregation (Figure 1a-c). Sectors were isolated, purified and inoculated on CM + benomyl (0.5 μL/mL) for ploidy evaluation (Miyamoto and others 2007). Sectors with mitotic stability when growing in the presence of benomyl were classified as haploids, after phenotypic characterization. On the other hand, diploid recombinants (Figure 1d) were identified and easily distinguished from haploid ones by their distinct growth and breakdown in the presence of benomyl, the haploidization agent. Aneuploid segregants, exhibiting reduced growth and mitotic instability higher than the diploid ones, were also identified. The number of color (yellow and white) recombinants per 100 colonies was measured and the induced Mitotic Segregation Index (MSI) calculated (the ratio

of recombinants per 100 treated to 100 untreated colonies). Index was used as a criterion to estimate the genotoxicity of *E. globulus* oil (Ziogas and Kalamarakis 2001).

3. RESULTS and DISCUSSION

3.1 *Chemical composition of the essential oil*

Table 1 shows the results of GC/MS analysis of *E. globulus* oil. Forty-five identified components accounted for 90.8% of the oil. The volatile oil consisted mainly of eucalyptol (49.05%), α-pinene (8.89%), β-pinene (1.5%), globulol (6.95%), α-eudesmol (1.12%), spathulenol (1.42%), γ-cadinene (1.45%), trans-β-elemenone (1.23%) and aromandendrene (2.33%), reaching together 73.9% of the oil. Although changes in the composition of *E. globulus* essential oils have been previously described (Silvestre and others 1997), the 1,8-cineole content of current oil sample is within values found in literature (Dagne and others 2000; Cimanga and others 2002).

3.2 *Cytotoxic of* E. globulus *essential oil*

Solid cultures of the A757//UT448 diploid strain, obtained in the presence of the oil (0.25 μL/mL and 0.5 μL/mL), reduced micelial growth, as the highest oil inhibitory effects observed after 48h of incubation for the two tested concentrations (Figure 2). The highest oil concentration (0.5 μL/mL) also affected the normal morphology and the spore production of the original A757//UT448 diploid strain (results not shown). In fact, the identification of color sectors growing on the diploid colonies and the estimation of the recombinogenic activity of the oil were difficult. Oil genotoxicity was thus evaluated by the lowest concentrations (0.0625 μL/mL, 0.125 μL/mL and 0.25 μL/mL).

Table 1. Essential oil composition from *Eucalyptus globulus*

	Compounds	Formula	WM	RT (min)	RI	%
01	α-Thujene	$C_{10}H_{16}$	136	3.314	922.60	0.17
02	α-Pinene	$C_{10}H_{16}$	136	3.467	930.25	8.89
03	Camphene	$C_{10}H_{16}$	136	3.797	946.75	0.06
04	Sabinene	$C_{10}H_{16}$	136	4.258	969.80	0.06
05	β-Pinene	$C_{10}H_{16}$	136	4.382	976.00	1.50
06	β-Myrcene	$C_{10}H_{16}$	136	4.602	987.00	0.36
07	α-Phellandrene	$C_{10}H_{16}$	136	5.049	1006.22	< 0.01
08	α-Terpinene	$C_{10}H_{16}$	136	5.327	1015.47	0.01
09	Ocymene	$C_{10}H_{14}$	134	5.540	1022.56	0.85
10	Limonene	$C_{10}H_{16}$	136	5.670	1026.89	0.03
11	Eucalyptol (1,8-cineol)	$C_{10}H_{18}O$	154	5.772	1030.28	49.05
12	γ-Terpinene	$C_{10}H_{16}$	136	6.506	1054.71	0.67
13	Terpinolene	$C_{10}H_{16}$	136	7.375	1083.63	0.09
14	*p*-Cymene	$C_{10}H_{12}$	132	7.566	1089.98	0.13
15	Linalool	$C_{10}H_{18}O$	154	7.890	1100.57	0.10
16	Isopentyl 2-methyl butanoate	$C_{10}H_{20}O_2$	172	8.104	1105.91	0.11
17	Endo-Fenchol	$C_{10}H_{18}O$	154	8.568	1117.48	0.28
18	Trans-Pinocarveol	$C_{10}H_{16}O$	152	9.342	1136.78	0.20
19	Borneol	$C_{10}H_{18}O$	154	10.566	1167.31	0.66
20	Terpin-4-ol	$C_{10}H_{18}O$	154	10.934	1176.48	0.28
21	*m*-Cymen-8-ol	$C_{10}H_{14}O$	150	11.286	1185.26	0.66
22	α-Terpineol	$C_{10}H_{18}O$	154	11.646	1194.24	2.04
23	Nerol	$C_{10}H_{18}O$	154	14.958	1249.65	0.11
24	Thymol	$C_{10}H_{14}O$	150	18.093	1300.18	0.31
25	Verbanol acetate	$C_{12}H_{20}O_2$	196	20.644	1345.55	0.36
26	Cyperene	$C_{15}H_{24}$	204	23.578	1397.72	0.94
27	Caryophyllene	$C_{15}H_{24}$	204	24.168	1409.56	0.20
28	β-gurjunene	$C_{15}H_{24}$	204	24.692	1420.41	0.30
29	Aromandendrene	$C_{15}H_{24}$	204	25.126	1429.39	2.33
30	allo-Aromandendrene	$C_{15}H_{24}$	204	26.165	1450.89	0.77
31	Guaiene	$C_{15}H_{24}$	204	27.573	1480.03	0.52
32	Viridiflorene	$C_{15}H_{24}$	204	27.713	1482.93	0.33
33	γ-Cadinene	$C_{15}H_{24}$	204	30.899	1561.13	1.45
34	Selina-3,7 (11)-diene	$C_{15}H_{24}$	204	31.178	1568.36	0.53
35	Spathulenol	$C_{15}H_{24}O$	220	31.538	1577.68	1.42
36	Caryophyllene oxide	$C_{15}H_{24}O$	220	31.713	1582.21	1.10
37	Globulol	$C_{15}H_{26}O$	222	31.866	1586.17	6.95
38	Viridiflorol	$C_{15}H_{26}O$	222	32.084	1591.82	1.78
39	β-elemeno	$C_{15}H_{22}O$	218	32.157	1593.71	0.86
40	Guaiol	$C_{15}H_{26}O$	222	32.317	1597.85	0.62
41	Trans-β-Elemenone	$C_{15}H_{22}O$	218	32.390	1599.23	1.23
42	Cis-Isolongifolanone	$C_{15}H_{24}O$	220	32.513	1608.75	0.80
43	Trans- Isolongifolanone	$C_{15}H_{24}O$	220	32.703	1623.47	0.36
44	γ- Eudesmol	$C_{15}H_{26}O$	222	32.849	1634.78	0.22
45	α-Eudesmol	$C_{15}H_{26}O$	222	33.162	1659.02	1.12
	Total identified					**90.80**

The identified constituents are listed in their order of elution from column CP-SIL 8 CB; RT - retention times; RI- retention indices (experimental data). Repetitive assays showed a less than 1% deviation.

3.3 *Recombinogenic activity of* E. globulus *essential oil*

The A757//UT448 diploid strain exhibited high mitotic instability in the presence of the oil, and mitotic color segregants growing on the green diploid colonies could be isolated (Figure 1b-c). Diploid segregants which had undergoing mitotic crossing-over, aneuploids generated by mitotic non-disjuction, and haploid segregants were recovered from the A757//UT448 diploid strain after treatment with *E. globulus* essential oil.

MSI values were not determined for aneuploid segregants due to their great mitotic instability. Unlike the *Achillea millefolium* oil, which induced a significant increase in the number of aneuploid segregants in *A. nidulans* diploid cells (Sant'Anna and others 2008), the *E. globulus* oil employed in present work showed no aneugenic activity (Table 2). Additionally, the haploid segregants, obtained after oil treatment (0.0625 μL/mL, 0.125 μL/mL and 0.25 μL/mL), showed parental phenotypes for their nutritional markers (results not shown), and explained the MSI values of less than or equal to 1.0 for haploid segregants (Table 2).

On the other hand, two of the three tested oil concentrations (0.125 μL/mL and 0.25 μL/mL) increased the number of diploid recombinants, when compared to the control (Table 2). Such recombinants, identified by the production of new white or yellow mitotic sectors when growing in CM + benomyl (Figure 1d), showed MSI values which were higher than 1.0 and significantly different from the respective control values (Table 2). It should be emphasized that the eucalyptol mass (116.18 mg) contained in the total volume of *E. globulus* oil (0.125 μL/mL) employed to obtain the oil's highest genotoxic concentration (0.25 μL/mL), corresponded, approximately, to 1/14 of the eucalyptol mass (1600 mg) contained in 1 Kg of hard candy (Burdock 1995).

Diploid strains of *A. nidulans*, heterozygous for genes determining conidial color, produce segregants expressing mutant colors, following mitotic crossing-over and haploidization.

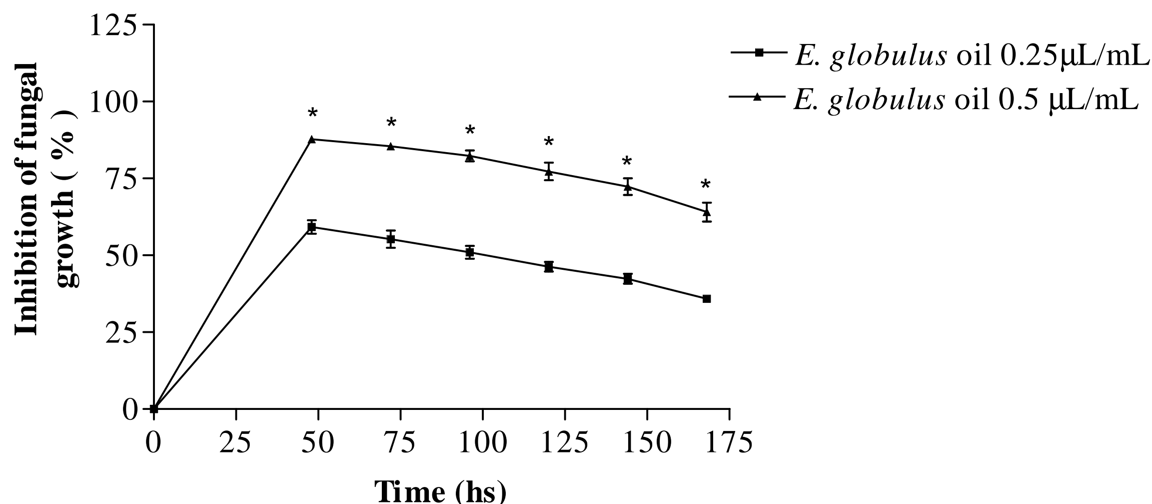

Figure 2. Growth inhibition of A757// UT448 diploid strain after treatment with *E. globulus* essential oil at 0.25 μL/mL and 0.5 μL/mL concentrations. Points represent average ± SD of five experiments. (*) Statistical differences between experiments performed with the two oil concentrations (Analysis of Variance, Bonferroni post test, $P < 0.05$).

The first process leads to the segregation (i. e., homozygosis) of all genetic markers distal to the position of exchange, and the second one, caused by a failure of regular chromosomes separation at mitosis, leads to the random assortment of whole chromosomes without crossing-over (Nga and Roper 1969). In current work, diploid recombinants were obtained by mitotic crossing-over, whereas the nuclei derived from the haploidization process, with a balanced haploid set, were the recovered haploid segregants. The remaining nuclei, showing high mitotic instability, were characterized as aneuploid segregants.

Data in current research indicate that the essential oil of *E. globulus* may act as a mitotic recombination inducer. This activity may be due to the induction of chromosome breaks by the oil, during the fungal growth, providing the condition for chromosome break-deletions or crossing-over to take place (Nga and Roper 1969; Ziogas and Kalamarakis 2001; Sant'Anna and others 2008).

Table 2. Genotoxicity of *E. globulus* oil in diploid cells of *Aspergillus nidulans.*

Oil concentrations (μl ml^{-1})[a]	**Number of colored segregants per 100 treated colonies**				
	Aneuploid Segregants	Diploid Recombinants		Haploid Segregants	
		NR[b]	MSI[c]	NS[d]	MSI[c]
0 (Control)	20	83	1.0	47	1.0
0.0625	20	121	1.5 (121/83)	28	0.6 (28/47)
0.125	19	290	3.5* (290/83)	20	0.9 (40/47)
0.25	22	378	4.6* (378/83)	74	1.5 (71/47)

[a]Mean of two replications; [b]number of recombinants; [c]Mitotic Segregation Index; [d]number of haploid segregants; [*]significantly different from control (Yates corrected Chi square, $P < 0.05$).

In the two-step model for carcinogenesis, in which both copies of a tumor-suppressor gene have to be defective (Bishop and Schiestl 2002; Honma 2005), the recombinogenic potential of the oil suggests that it may act as an inducer of the secondary hit (mitotic recombination) which imparts the expression of germline or sporadic mutations (the first hit), previously masked by the presence of the normal allele. Whereas present results point to the need to assess the recombinogenic potential of the *E. globulus* essential oil in mammalian cells, they also suggest that the use of the oil as a flavour ingredient in foods must be done with caution.

CONCLUSIONS

Eucalyptus globulus oil, containing 1,8-cineol (eucalyptol), α-pinene, β-pinene, globulol, α-eudesmol, spathulenol, γ-cadinene, trans-β-elemenone and aromandendrene as its major chemical compounds, was found to increase (i) the mitotic instability of the original A757//UT448 diploid strain, (ii) the frequency of diploid recombinants and (iii) MSI values for diploid segregants, when used at 0.125 μL/mL and 0.25 μL/mL concentrations in *A. nidulans*

diploid cells. Results suggest that the use of *E. globulus* oil in food industry must be done with caution. Further studies should be conducted to clarify the recombinogenic activity of *E. globulus* essential oil in mammalian cells.

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REFERENCES

Ali NAA, Wurster M, Arnold N, Teichert A, Schmidt J, Lindequist U, Wessjohann L. 2008. Chemical Composition and Biological Activities of Essential Oils from the Oleogum Resins of Three Endemic Soqotraen *Boswellia* Species. Rec Nat Prod 2: 6-12.

Adams RP.1995. Identification of Essential Oils by Gas Chromatography and Mass Spectroscopy. Carol Stream: Allured Publishing Corporation, Illinois, USA. p. 469.

Bergen GG, Morris NR. 1983. Kinetics of the nuclear division cycle of *Aspergillus nidulans.* J Bacteriol 156: 155-160.

Bishop AJR, Schiestl RH. 2002. Homologous recombination and its role in carcinogenesis. J Biomed Biotechnol 2: 75-85.

Burdock, GA. 1995. Fenaroli's handbook of flavor ingredients. 3rd. Edition. CRC Press, Boca Raton, FL. p.121-122.

Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. Int J Food Microbiol 94: 223– 253.

Cermelli C, Fabio A, Fabio G, Quaglio P. 2008. Effect of eucalyptus essential oil on respiratory bacteria and viruses. Curr Microbiol 56: 89–92.

Cimanga K, Kambu K, Tona L, Apers S, De Bruyne T, Hermans N, Totté J, Pieters L, Vlietinck AJ. 2002. Correlation between chemical composition and antibacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. J Ethnopharmacol 79: 213-220.

Dagne E, Bisrat D, Alemayehu M, Worku T. 2000. Essential oils of twelve *Eucalyptus* species from Ethiopia. JEOR 12: 467-470.

De Vicenzi M, Silano M, De Vicenzi A, Maialetti F, Scazzochio B. 2002. Constituents of aromatic plants: eucalyptol. Fitoterapia 73: 269-275

Festa RS, Meadows AT, Boshes R. A. 1979. Leukemia in a black child with Bloom's syndrome: somatic recombination as a possible mechanism for neoplasia. Cancer 44: 1507-1510.

Fisher K, Phillips C. 2008. Potential antimicrobial uses of essential oils in food: is citrus the answer? Trends Food Sci Technol 19: 156-164.

Gomes-Carneiro MR, Felzenszwalb I, Paumgartten FR. 1998. Mutagenicity testing (+/)--camphor, 1,8-cineole, citral, citronellal, (-)-menthol and terpineol with the Salmonella/microsome assay. Mutat Res 416: 129-136.

Honma M. 2005. Generation of loss of heterozygosity and its dependency on p53 status in human lymphoblastoid cells. Environ Mol Mutagen 45 162-176.

Jirkova K, Mandys V, Gispen WH, Bär PR. 2006. Cisplatin-induced apoptosis in cultures of human Schwann cells. Neurosci Lett 392*:* 22-26.

Juergens UR, Dethlefsen U, Steinkamp G, Gillissen A, Repges R, Vetter H. 2003. Anti-inflammatory activity of 1.8-cineol (eucalyptol) in bronchial asthma: a double-blind placebo-controlled trial. Respir Med 97: 250-256.

Kaneshima EN, Castro-Prado MAA. 2005. Benznidazole-induced genotoxicity in diploid cells of *Aspergillus nidulans.* Mem Inst Oswaldo Cruz 1000: 325-329.

Koitabashi R, Suzuki T, Kawazu T, Sakai A, Kuroiwa H, Kuroiwa T. 1997. 1,8-cineole inhibits root growth and DNA synthesis in the root apical meristem of *Brassica campestris* L. J Plant Res 110: 1-6.

Miyamoto CT, Sant'Anna JR, Franco CCS, Castro-Prado MAA. 2007. Genotoxicity (mitotic recombination) of the cancer chemotherapeutic agents Cisplatin and Cytosine arabinoside in *Aspergillus nidulans*. Food Chem Toxicol 45: 1091-1095.

Moteki H, Hibasami H, Yamada Y, Katsuzaki H, Imai K, Komiya T. 2002. Specific induction of apoptosis by 1,8-cineole in two human leukemia cell lines, but not in human stomach cancer cell line. Oncol Rep 9: 757-760.

Moynahan ME, Jasin M. 1997. Loss of heterozygosity induced by a chromosomal double-strand break. Proc Natl Acad Sci USA 94: 8988–8993.

Nga BH, Roper JA. 1969. A system generating spontaneous intrachromosomal changes at mitosis in *Aspergillus nidulans*. Genet Res 14: 63-70.

Nielsen JB. 2006. Natural oils affect the human skin integrity and the percutaneous penetration of benzoic acid dose-dependently. Basic Clin Pharmacol Toxicol 98: 75-581.

Sacchetti G, Maietti S, Muzzoli M, Scaglianti M, Manfredini S, Radice M, Bruni R. 2005. Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods. Food Chem 91: 621–632.

Sant'Anna JR, Franco CCS, Miyamoto CT, Cunico MM, Miguel OG, Côcco LC, Yamamoto CI, Junior CC, Castro-Prado MAA. 2008. Genotoxicity of *Achillea millefolium* essential oil in diploid cells of *Aspergillus*. Phytother Res DOI 10.1002/ptr.2596

Santos SJ, Takahashi CS, Natarajan AT. 1994. Cytogenetic effects of the antichagasic benznidazole on human cells in vitro. Mutat Res 320: 305-314.

Silva J, Abebe W, Sousa SM, Duarte VG, Machado MIL, Matos FJA. 2003. Analgesic and anti-inflammatory effects of essential oils of Eucalyptus. J Ethnopharmacol 89: 277-283.

Silvestre AJD, Cavaleiro JAS, Delmond B, Filliatre C, Guy Bourgeois G. 1997 Analysis of the variation of the essential oil composition of *Eucalyptus globulus* Labill. from Portugal using multivariate statistical analysis. Ind Crops Prod 6 :27-33.

Stürzbecher HW, Donzelmann B, Henning W, Knippschild U, Buchhop S. 1996. p53 is linked directly to homologous recombination processes via RAD51/RecA protein interaction. EMBO J 15: 1992-2002.

Thyagarajan B, Campbell C. 1997. Elevated Homologous Recombination Activity in Fanconi Anemia Fibroblasts. J Biol Chem 272: 23328-23333.

Uemura M, Hata G, Toda T, Weine FS 1997. Effectiveness of EUC and d-limonene as gutta-percha solvents. J Endod 23: 739-741.

van Den Dool, H, Kratz PD. 1963. A Generalization of the Retention Index System Including Linear Temperature Progammed Gas-Liquid Partition Chromatography. J Chromatogr 11: 463-471.

Viuda-Martos Y, Ruiz-Navajas J, Fernández-López J, Pérez-Álvarez. 2008. Antifungal activity of lemon (*Citrus lemon* L.), mandarin (*Citrus reticulate* L.), grapefruit (*Citrus paradise* L.) and orange (*Citrus sinensis* L.) essential oils. Food Control 19: 1130-1138.

Ziogas BN, Kalamarakis AE. 2001. Phenylpyrrole fungicides: Mitotic instability in *Aspergilus nidulans* and resistance in *Botrytis cinerea*. J Phytopathol 149: 301-308.

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